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14. ABSTRACT

The androgen-dependent prostate cancer cell LNCaP undergoes apoptosis in response to PMA treatment, a process primarily mediated by one of the PKC isoforms PKCδ. Tyrosine phosphorylation, as a unique property and regulatory mechanism of PKCδ, has been studied over years but never in prostate cancer cells. In this report we identified PKCδ became Tyr phsophorylated at site Ty311 in response to PMA stimulation. But mutation of Tyr 311 affect neither PKCδ translocation, nor the apoptotic effect of PMA. By using adenovirus infection approach, we found that the other five sites, Tyr52, Tyr64, Tyr155, Tyr187 and Tyr565 are not relevant to the apoptosis of LNCaP cells. We eventually developed methods to analyze the signaling events controlled by Tyr phosphorylation. Etoposide, an anti-cancer agent whose effect is dependent on PKCδ, does not induce Tyr phosphorylation of PKCδ in LNCaP cells, indicating different drug effects might rely on different mechanisms. In summary, in this study we found the Tyr sites we examined so far are irrelevant to the apoptotic effect of PMA in LNCaP cells. However, more study will be needed to make a final conclusion on the role of PKCδ Tyr phosphorylation in the apoptosis induced by PMA.

15. SUBJECT TERMS
PKC, prostate cancer, tyrosine phosphorylation, apoptosis

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Introduction

The protein kinase C (PKC) family comprises a number of related serine-threonine kinases that regulate numerous intracellular pathways involved in the control of cell cycle progression, differentiation, survival, transformation and apoptosis (1). The androgen-dependent prostate cancer cell line LNCaP undergoes apoptosis in response to phorbol 12-myristate 13- acetate (PMA) treatment, a process in which one of the PKC isoforms, PKCδ plays a central role (2). There is emerging evidence that PKCδ also mediates the apoptotic effect of many anticancer drugs, such as TNFα, Ginsenoside Rh2, and etoposide (3-5). Early studies have shown that PKCδ becomes phosphorylated on tyrosine (Tyr) residues upon acute stimulation with H_2O_2, PMA, EGF, or PDGF, a unique property of PKCδ (6). So far nine Tyr phosphorylation sites have been identified across its regulatory domain, catalytic domain, and hinge region. Tyrosine phosphorylation regulates PKCδ activity and impacts its cellular functions (6-7). For instance, in C6 glioma cells, phosphorylation in Tyr64 and Tyr187 is essential for etoposide-induced apoptosis (5). In cardiomyocytes, PMA stimulates phosphorylation of Tyr311 and increases PKCδ kinase activity (8). No study has been done in prostate cancer cells regarding PKCδ Tyr phosphorylation. Our studies set to address how Tyr phosphorylation regulates the effect of PMA and etoposide in androgen-dependent LNCaP prostate cancer cells, to determine the specific Tyr phosphorylation sites upon treatment with PMA and etoposide, the contribution of phosphorylated Tyr to the apoptotic effect of those agents, and the downstream signaling events controlled by tyrosine phosphorylation. We expect that our studies will provide deep insight into the mechanisms that regulate the effect of anticancer drugs, and will eventually contribute to the understanding on how anti-cancer agents exert their effects.

Body

1. To analyze in LNCaP cells the Tyr phosphorylation of PKCδ in IPs after PMA and etoposide treatment. To determine if PKCδ becomes Tyr phosphorylated in response to PKC activation, we treated LNCaP cells with the phorbol ester PMA (PKC

activator, 100 nM, 1 h), performed immunoprecipitation (IP) with an anti-PKCδ specific antibody and then analyzed by Western blot with an anti-phospho-Tyr antibody. We found that PMA induced strong Tyr phosphorylation of PKCδ (Fig. 1). We also treated cells with etoposide (300 μM), and took samples at different time points to analyze Tyr phosphorylation in IPs followed by Western blot. Interestingly, etoposide did not induce Tyr phosphorylation. These results suggest that there are different regulatory mechanisms for PKCδ depending on the distinct apoptotic agents used.

2. To analyze by Western blot Tyr phosphorylation of PKCδ after PMA treatment using site-specific antibodies. We treated cells with PMA (100 nM, 1 h) and used different site-specific antibodies to map phosphorylation sites. The following Tyr site-specific antibodies were used: anti-Tyr52, anti-Tyr155 anti-Tyr311 (kind gifts from Chaya Brodie, Henry Ford Hospital, Detroit). For all of the site-specific antibodies tested, we only detected a strong band with the Tyr311 antibody (Fig. 2). Since the pre-existing commercially available phospho-Tyr PKCδ antibodies do not cover all of the Tyr sites of PKCδ, mass spectrometry might be needed in order to identify other potential phosphorylation sites. In conclusion, we identified Tyr311 as a relevant Tyr site in response to PMA.

3. To analyze the apoptotic effect of PMA on cells infected with AdVs of various PKCδ Tyr mutants. LNCaP is a cell line that shows very low transfection efficiency. Therefore, we decided to use adenoviral infection as a delivery approach. There are 9 potential Tyr sites on PKCδ. First, we used two different adenoviruses (AdVs): WT-PKCδ (wild type) and PKCδ5 (PKCδ with the five Tyr sites Tyr52, Tyr64, Tyr155, Tyr187 and Tyr565 replaced by Phe, a kind gift from Chaya Brodie). To achieve an optimal overexpression with minimum detachment of the cells that we normally observe at high MOIs, we performed multiplicities of infection (MOI) test by infecting cells with each AdV at different MOI, and found that at an MOI of 3 pfu/cell, both AdVs cause significant overexpression (Fig. 3).

We infected LNCaP cells with AdVs for either WT-PKCδ or PKCδ5, and we used LacZ AdV as a control. We carried out a dose-response curve for PMA (0-30 nM, 1 h) and

determined apoptosis by DAPI staining 24 h later. In agreement with a previous report from our laboratory (2), WT-PKCδ AdV significantly potentiated PMA-induced apoptosis at 3 nM and 10 nM PMA when compared to control LacZ AdV, but they both showed the same apoptotic ratio at 30 nM of PMA (~25%) which is the saturation point (Fig. 4). However, the PKCδ5 AdV-infected cells showed the same apoptotic ratio as WT-PKCδ AdV-infected cells, which argues that none of the 5 Tyr sites (Tyr52, Tyr64, Tyr155, Tyr187 and Tyr565) are relevant for the PMA effect. Since we found that etoposide does not cause Tyr phosphorylation in LNCaP cells, we decided to focus only on the PMA effect.

4. To analyze PKCδ translocation with GFP-tagged PKCδ Tyr mutants. As an alternative approach to adenoviral infection, we used Amaxa Nucleofector system to transfect LNCaP cells. The following PKCδ constructs were used: WT-PKCδ-GFP, PKCδ5-GFP, and Tyr311-GFP. Forty-eight h following transfection, the cells were treated with 1μM PMA and collected at 0, 10, and 20 min after treatment. Cells were fixed and subjected to confocal microscopy. We found that both the PKCδ5 and Tyr 311 mutant translocated to plasma membrane after PMA treatment (Fig. 5), and the translocation pattern was completely the same as wild type. Therefore, tyrosine phosphorylation does not affect PKCδ translocation.

5. To analyze the apoptotic effect of PMA on cells transfected with various GFP-tagged PKCδ Tyr mutants by flow cytometry. We transfected LNCaP cells with either a control GFP, or GFP tagged PKCδ Tyr mutants by using Amaxa Nucleofector. The following PKCδ constructs were used: WT-PKCδ-GFP, PKCδ5-GFP, and Tyr311-GFP. Forty-eight h following transfection, cells were treated with either 3 or 30 nM PMA for 1h. Twenty-four h later, cells were collected and subjected to flow cytometry analysis following PI staining to access their apoptotic ratio both in green (transfected) cells and dark (non-transfected) cells. In agreement with a previous report from our laboratory (2), WT-PKCδ-GFP significantly potentiated PMA-induced apoptosis at 3 nM PMA when compared to control GFP, but they both showed the same apoptotic ratio at 30 nM of PMA (~25%) which is the saturation point (Fig. 6). However, both PKCδ5-GFP and Tyr

311-GFP had a potentiation effect on PMA induced apoptosis. In the mean while, the untransfected (dark) cells from all samples showed identical apoptotic ratio as GFP control transfected cells. The result indicated that none of the Tyr sites that we examined (Tyr52, Tyr64, Tyr155, Tyr187, Ty311 and Tyr565) are relevant to the PMA effect, although we proved that Tyr311 is Tyr phoshorylated in response to PMA stimulation.

6. To determine how Tyr phosphorylation influences the apoptotic effect of CM. In a recent paper we identified that PKCδ-mediated apoptosis involves the autocrine secretion of death factors (9), in which TNFα and Trail are two major components. The secretion of the death factors to the conditioned medium (CM) is indispensable to the apoptosis, since constant removal of the CM from PMA treated cells resulted in impaired apoptosis (9). To determine whether Tyr phosphorylation influences the apoptotic effect of CM, we first proposed to use WT-PKCδ and PKCδ5 AdV to infect the cells, collect the CMs from those cells, and then examine the abilities of those CMs to induce apoptosis. However, we found the PKCδ5 AdV overexpression had the same effect on PMA induced apoptosis as WT- PKCδ5 AdV and ruled out the contribution of the 5 Tyr sites (Tyr52, Tyr64, Tyr155, Tyr187 and Tyr565) to apoptosis. To examine whether there are other Tyr sites involved, we decided to test whether the Tyr kinase inhibitor genistein has any effect on PMA-induced apoptosis. We treated cells with genistein (100 μM) for different times and then treated with either 100 nM PMA or vehicle for 1 h. CM was collected from those cells 24 h later and applied to fresh LNCaP cells. As shown in Fig. 7, CM from PMA-treated cells, but not from vehicle-treated cells, caused a marked apoptotic response. We found that pretreatment with genistein for > 6 h significantly reduced the ability of CM from PMA-treated cells to induce apoptosis. These results indicate that Tyr phosphorylation might be important for the apoptotic activity of the CM.

FUTURE DIRECTIONS

MS spectrum will be employed to examine the Tyr sites that we have not studied to access whether they contribute to apoptosis.

KEY RESEARCH ACCOMPLISHEMENTS

1. We found that PMA stimulation induces the Tyr phosphorylation of PKCδ in LNCaP cells, while etoposide treatment does not cause this effect.
2. We found that PMA causes Tyr phosphorylation in Tyr311 in LNCaP cells.
3. We established the methodology to overexpress PKCδ Tyr mutants in LNCaP cells by adenoviral infection.
4. We found that Tyr52, Tyr64, Tyr155, Tyr187, and Tyr565 are irrelevant to PMA-induced apoptosis in LNCaP cells.
5. We developed the methodology to measure apoptosis in GFP-transfected green cells vs. untransfected cells by flow cytometry.
6. We found Tyr311 mutant affect neither PKCδ translocation nor apoptosis in response to PMA in LNCaP cells.
7. We provided preliminary results with a pharmacological inhibitor of Tyr kinases (genistein) that indicate that Tyr phosphorylation contribute to the apoptotic activity of the CM from PMA-treated LNCaP cells.

Reportable outcomes

Xiao L, Caino MC, von Burstin VA, Oliva JL, Kazanietz MG. Phorbol ester-induced apoptosis and senescence in cancer cell models. Methods Enzymol. 446:123-39 (2008).

Conclusions

In the last two years we completed experiments to address whether PMA and etoposide treatment induce Tyr phosphorylation of PKCδ in prostate cancer cells and how Tyr phosphorylation influences apoptosis. We found that PMA induces Tyr phosphorylation of PKCδ at Tyr311. This is the first report showing Tyr phosphorylation of PKCδ in prostate cancer cells in response to PKC activation. Regarding the identity of the Tyr phosphorylation studies, we ruled out the relevance of the 5 sites (Tyr52, Tyr64, Tyr155, Tyr187 and Tyr565) in the apoptotic response of PMA. However, we found mutation of Tyr311 to Ala affect neither PKCδ translocation nor the apoptotic effect of PMA in LNCaP cells, which means Tyr311 is not relevant to apoptosis, though it is a PMA responsive site. Since there are 9 potential Tyr phosphorylation sites on PKCδ, and the currently available commercial antibodies do not cover all of the phosphorylation

sites, we cannot rule out the possibility that there are other Tyr sites involved. MS spectrum analysis, and possibly, generation of new Tyr mutants will be need to address these questions and make a final conclusion about whether Tyr phosphorylation of PKCδ is relevant to apoptosis. Meanwhile, we developed a series of methodologies including IP, adenoviral infection, flow cytometry, and cytokine measurement, which paved ways for further research on the apoptotic effect and signaling events controlled by PKCδ in prostate cancer cells.

We also found that etoposide does not induce Tyr phosphorylation of PKCδ, though there is evidence showing its effect is dependent on PKCδ activity in other cell types. For example, in glioma cells, etoposide-induced apoptosis is dependent on PKCδ (5), which suggests marked cell type differences. Therefore, we conclude that different anti-cancer agents rely on different regulatory mechanisms via PKCδ to cause cell death, and that distinct mechanisms probably operate in different cell types.

In the future our research will focus on the following directions: to identify other possible Tyr phosphorylation sties in response to PMA stimulation by MS spectrum; if there is any, then to generate new GFP-tagged Tyr mutants, to identify the contribution of those sites to apoptosis; to develop the methodologies and analyze how Tyr phosphorylation controls CM secretion and downstream signaling events. These studies will provide important information on our understanding of the effects of apoptotic agents in prostate cancer cells.

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Abbreviations

PKC: Protein kinase C

PMA: Phorbol 12-myristate 13-acetate

GFP: Green fluorescence protein

AdV: Adenovirus

MOI: Multiplicity of infection

CM: Conditioned medium

IP: Immunoprecipitation

WT: Wild-type

DAPI: 4', 6-diamidino-2-phenylindole

TNFα: Tumor necrosis factor α

ELISA: Enzyme-linked Immunosorbent Assay

Tyr: Tyrosine

Appendix

7 figures

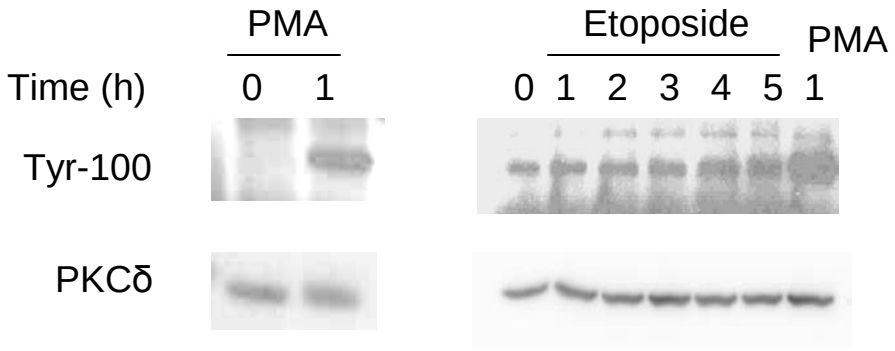

Fig. 1: PMA stimulation, but not etoposide, induces Tyr phosphorylation of PKCδ in LNCaP cells. Cells were treated with 100 nM PMA for 1 h or 300 µM etoposide for the indicated times. Cells were subject to IP with an anti-PKCδ antibody and Tyr phosphorylation was determined in IPs using an anti-phosphoTyr antibody (Tyr-100) or an anti-PKCδ antibody as a loading control. In the case of etoposide stimulation, PMA stimulation was used as a positive control. Similar results were found in 3 independent experiments.

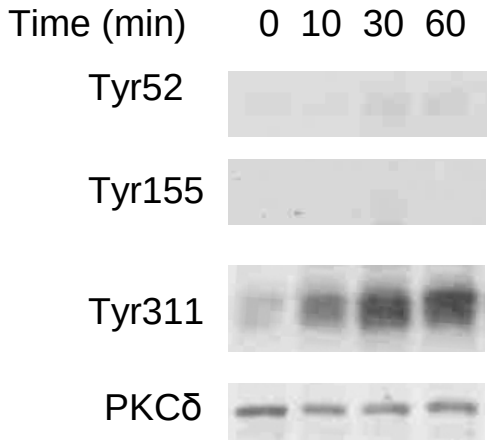

Fig. 2: PMA stimulation induces Tyr phosphorylation of PKCδ at Tyr311 in LNCaP cells. Cells were treated with PMA for the indicated times and subject to Western blot with different site-specific anti-PKCδ phospho-Tyr antibodies and total PKCδ as a loading control. Similar results were founded in 3 independent experiments.

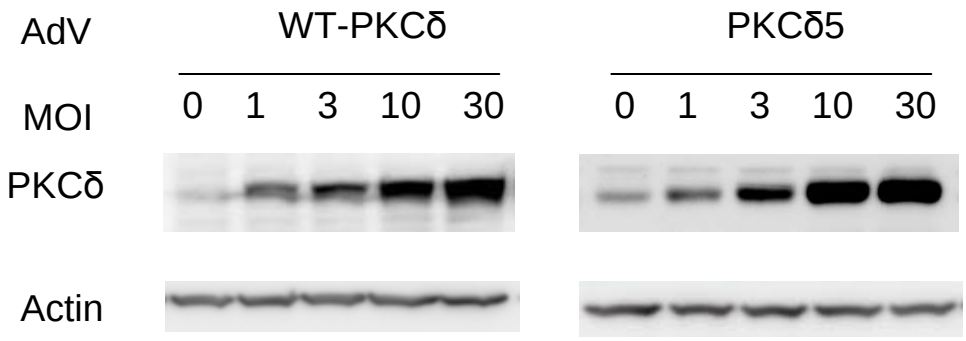

Fig. 3: Adenoviral delivery of PKCδ AdVs into LNCaP cells. Cells were infected with WT-PKCδ or PKCδ5 AdV for 16 h. Forty-eight hours later, cells were collected and PKCδ expression was analyzed by Western blot. Actin was used as a loading control. Both WT-PKCδ and PKCδ5 AdVs induce over expression at MOI 3 pfu/cell and larger. Similar results were observed in 3 independent experiments.

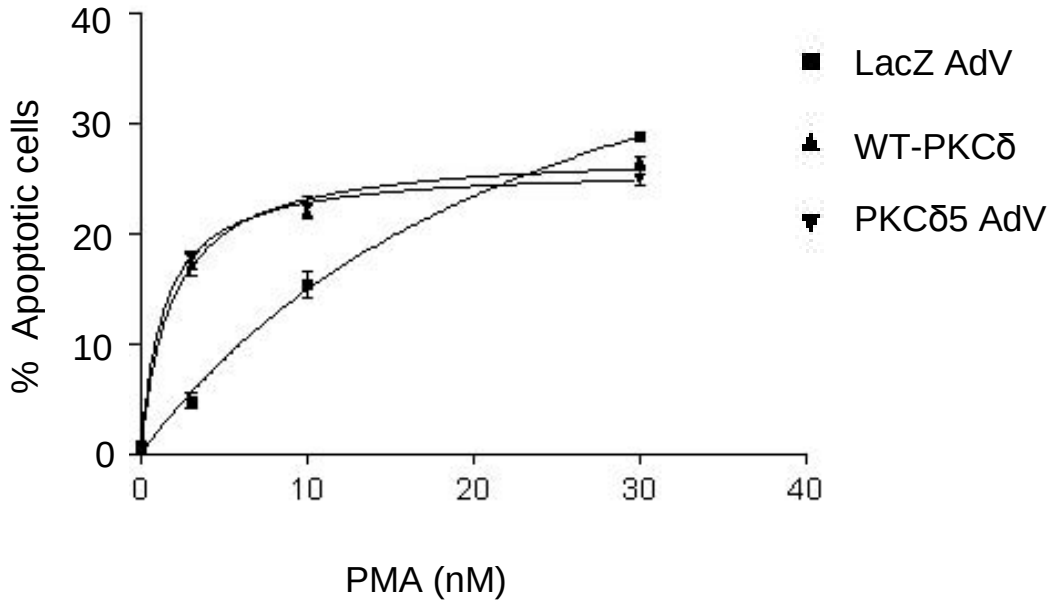

Fig. 4: Both WT-PKCδ and PKCδ5 AdVs potentiate PMA-induced apoptosis to the same extent. Cells were infected with LacZ, WT-PKCδ or PKCδ5 AdV for 16 h. Forty eight h later cells were treated with PMA at the indicated concentrations for 1 h. Cells were collected 24 h later to determine apoptosis. Date was expressed as mean ± S.D. (n=3). Similar results were observed in 3 independent experiments.

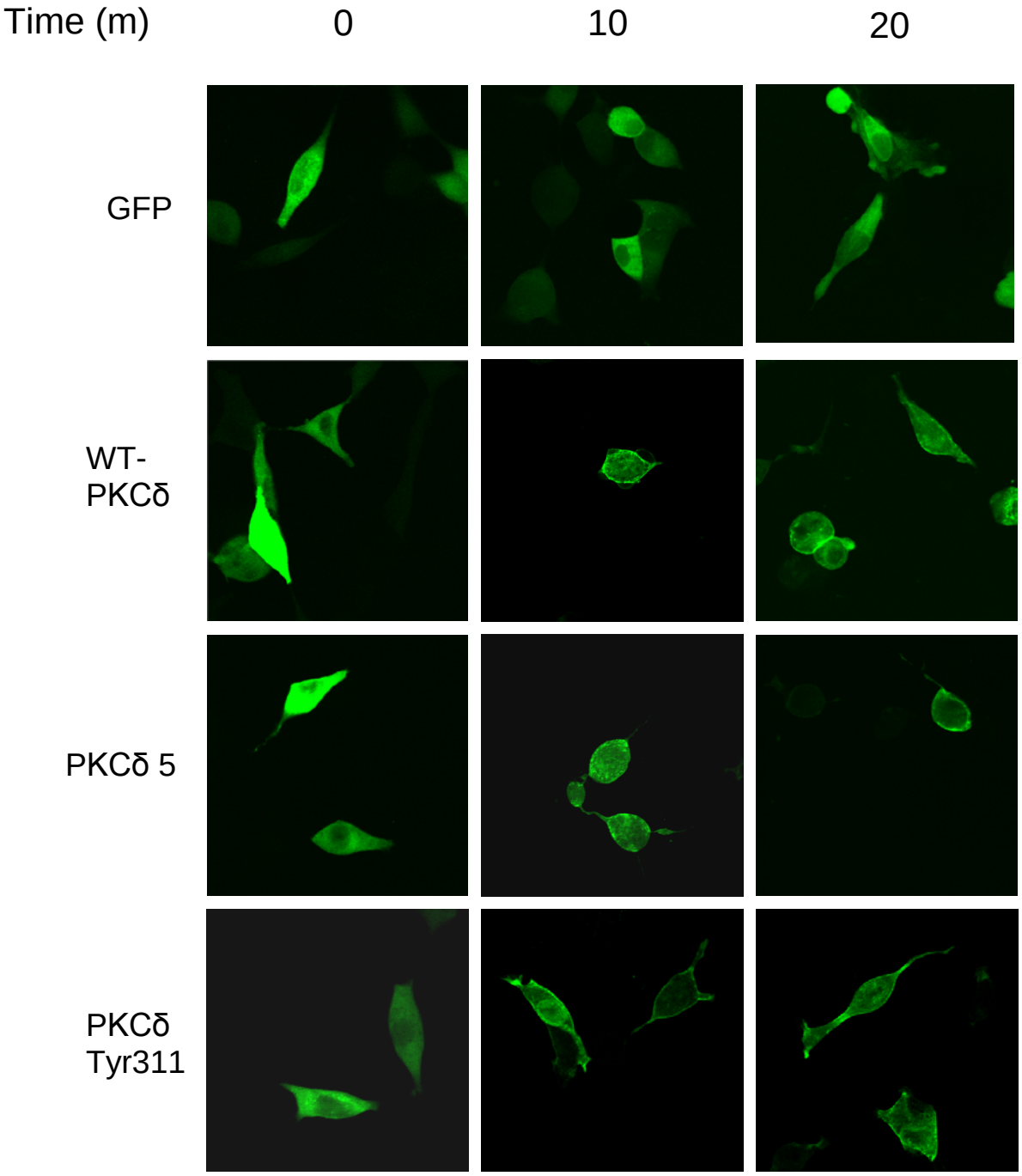

Fig. 5: PKCδ 5 and PKCδ Tyr311 do not affect PKCδ translocation in response to PMA. Cells were transfected with either a GFP control plasmid or WT-PKCδ -GFP, PKCδ5-GFP, and Tyr311-GFP. Forty-eight hours after transfection, cells were stimulated with 1μM PMA and collected at indicated times. Cells were fixed and subjected to confocal fluorescent microscopy.

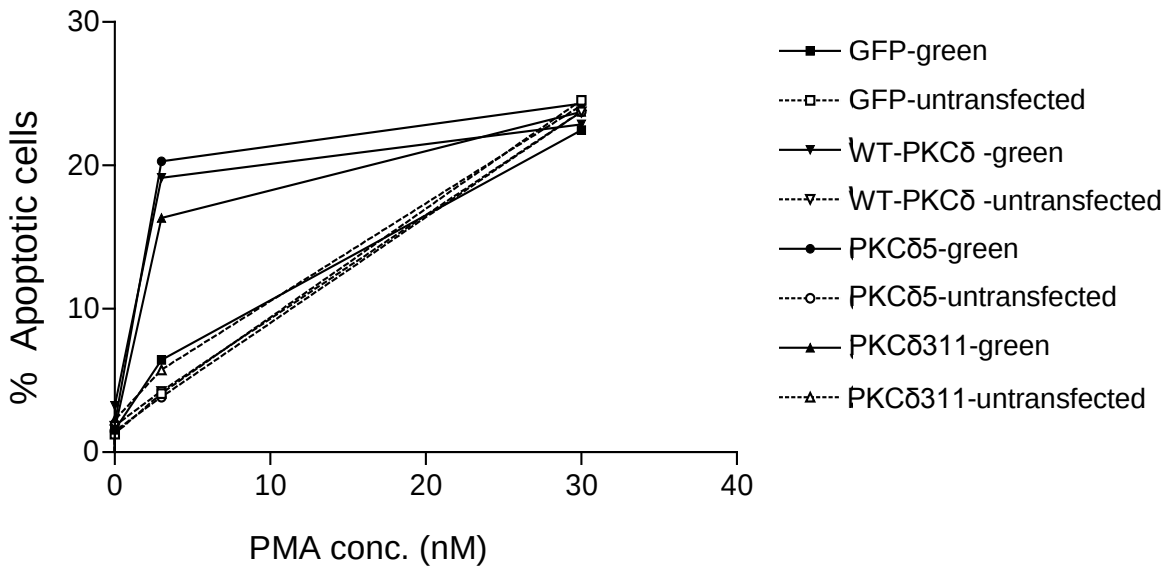

Fig. 6: WT-PKCδ-GFP, PKCδ5-GFP and PKCδ311-GFP potentiate PMA-induced apoptosis to the same extent. Cells were transfected with GFP, WT-PKCδ-GFP or PKCδ5-GFP. Forty-eight h later, cells were treated with either 3 or 30 nM PMA for 1h. Twenty-four h later, cells were collected and subjected to flow cytometry analysis following PI staining to determine apoptosis. Similar results were observed in 3 independent experiments.

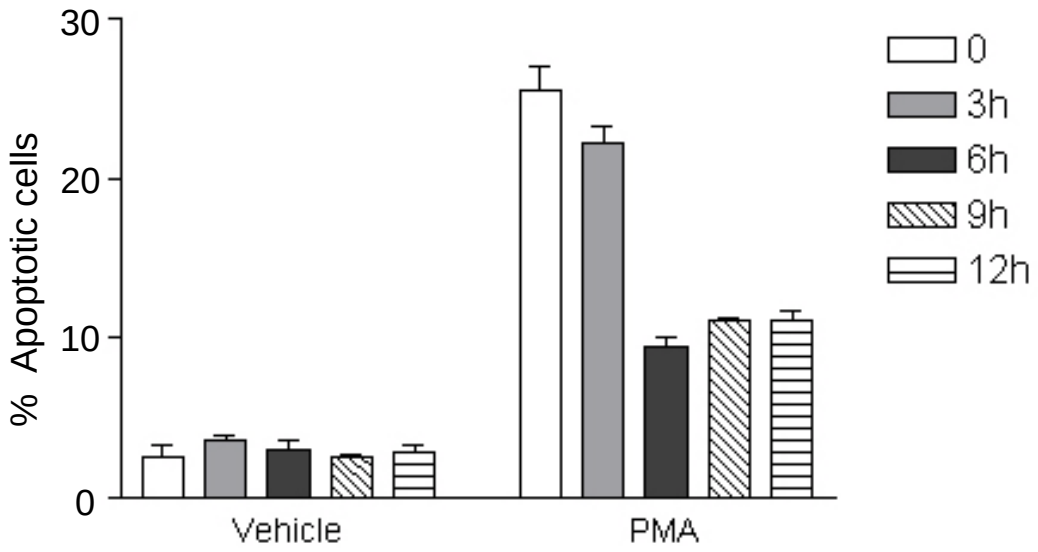

Fig. 7: Genistein blocks the apoptotic effect of CM from PMA-treated LNCaP cells. Cells were pretreated with 100 µM genistein for the indicated times and then were treated with either vehicle or PMA (100 nM) for 1 h. CMs were collected 24 h later and applied to fresh LNCaP cells. Apoptosis was determined 24 h later.